INFECTION AND IMMUNITY, June 2002, p. 2820–2827 Vol. 70, No. 6
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.6.2820–2827.2002
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Regulation of Antigen-Specific Immunoglobulin G Subclasses in Response to Conserved and Polymorphic *Plasmodium falciparum* Antigens in an In Vitro Model

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Received 11 November 2001/Returned for modification 15 December 2001/Accepted 25 February 2002

Cytophilic antibodies (Abs) play a critical role in protection against *Plasmodium falciparum* blood stages, yet little is known about the parameters regulating production of these Abs. We used an in vitro culture system to study the subclass distribution of antigen (Ag)-specific immunoglobulin G (IgG) produced by peripheral blood mononuclear cells (PBMCs) from individuals exposed to *P. falciparum* or unexposed individuals. PBMCs, cultivated with or without cytokines and exogenous CD40/CD40L signals, were stimulated with a crude parasite extract, recombinant vaccine candidates derived from conserved Ags (19-kDa C terminus of merozoite surface protein 1 [$MSP1_{19}$], R23, and PfEB200), or recombinant Ags derived from the polymorphic Ags MSP1 block 2 and MSP2. No *P. falciparum*-specific Ab production was detected in PBMCs from unexposed individuals. PBMCs from donors exposed frequently to *P. falciparum* infections produced multiple IgG subclasses when they were stimulated with the parasite extract but usually only one IgG subclass when they were stimulated with a recombinant Ag. Optimal Ab production required addition of interleukin-2 (IL-2) and IL-10 for all antigenic preparations. The IgG subclass distribution was both donor and Ag dependent and was only minimally influenced by the exogenous cytokine environment. In vitro IgG production and subclass distribution correlated with plasma Abs to some Ags ($MSP1_{19}$, R23, and MSP2) but not others (PfEB200 and the three MSP1 block 2-derived Ags). Data presented here suggest that intrinsic properties of the protein Ag itself play a major role in determining the subclass of the Ab response, which has important implications for rational design of vaccine delivery.

Passive transfer of hyperimmune immunoglobulin G (IgG) to malaria patients has shown that antibodies (Abs) play a key role in protection against *Plasmodium falciparum* blood stages (12, 25, 30). In vitro studies have indicated that efficient parasite destruction is achieved through interaction of Abs with monocytes. Binding of Abs to an infected red blood cell results in opsonization of the infected cell (24), and Ab-dependent cellular inhibition of parasite growth is triggered by the binding of an IgG-merozoite complex to monocytes (5, 6, 40). Both mechanisms have been reported to involve IgG1 and IgG3 but not IgG2, which is normally noncytophilic (4, 8, 23). The efficiency of Ab-dependent cellular inhibition of parasite growth depends on the relative proportion of parasite-specific IgG1 and IgG3 compared to IgG2 (4). The data indicate that the subclass distribution of IgG Abs reacting with merozoite surface antigens (Ags) is an important parameter for protection against *P. falciparum* blood stages. In order to develop efficient blood stage vaccines, we need to better understand anti-*P. falciparum* IgG subclass production and/or switching.

Identification of the parasite and host factors affecting Ab production by B cells might indicate ways in which vaccine-induced immune responses can be directed to ensure terminal differentiation of B cells for production of the most appropriate IgG subclasses. The molecular mechanisms driving the production of different classes and subclasses of Abs in individuals acquiring immunity to malaria are still largely unknown. To address this question, we studied the in vitro production of IgG to a crude *P. falciparum* parasite extract and to recombinant proteins derived from blood stage Ags. These Ags include polymorphic Ags located on the merozoite surface, such as MSP1 block 2 and MSP2, as well as conserved Ags associated with the red blood cell membrane, namely, Ag 332 (PfEB200) and Ag R45 (R23). The plasma Ab responses to these Ags in human populations exposed to *P. falciparum* infection have been documented in several seroepidemiological studies (10, 11, 32–35, 41). Some Ags have conferred substantial protection in vaccination trials of nonhuman primates (36).

Peripheral blood mononuclear cells (PBMCs) were collected from naive individuals, from subjects residing in urban areas where malaria was hypoendemic who were probably poorly immune, and from putatively immune adults living in

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Senegalese villages where malaria was meso- and holoendemic. PBMCs were cultured with malaria Ag in the presence or absence of various cytokines and costimulatory signals (sCD40L), and IgG production was monitored by enzyme-linked immunosorbent assay (ELISA) of cell culture supernatants.

MATERIALS AND METHODS

Blood donors. Three groups of volunteer blood donors were studied. The first group consisted in 87 immune healthy Senegalese males and nonpregnant females with no history of clinical malaria in the 6 months preceding the study. These >20-year-old adults lived in Dielmo and Ndiop in southwest Senegal, where malaria is holoendemic and mesoendemic, respectively. They had experienced life-long exposure to means of 200 and 20 infective bites per year, respectively (44, 45).

The second group of donors comprised 15 healthy young males adults donating blood at the blood bank of the Hôpital Principal de Dakar. These individuals had not experienced any clinical malaria within the proceeding since malaria endemicity in the Dakar area is low, with less than 1 infective bite per year (43). The members of this group were likely to have had limited previous experience of malaria. Blood samples from members of the first two groups were checked for the absence of circulating parasites by means of the quantitative buffy coat QBC test (Becton-Dickinson/H2F, Abijan, Ivory Coast).

The third group of blood donors consisted of seven healthy adult European expatriates who had recently settled in an area where malaria is endemic and had had no recent or past exposure to *P. falciparum.* These donors are referred to below as unexposed individuals.

Blood sampling was performed after informed consent was obtained. The protocols for blood sampling were reviewed and approved by the Senegalese national ethical committee. Ethical clearance was issued for withdrawal of no more than 25 ml of blood, and the volumes obtained ranged from 15 to 25 ml per individual.

Ag preparations. The crude Ag preparation consisted of a lysate of in vitro, mature, schizont-enriched, *P. falciparum*-infected red blood cells (2).

The following recombinant proteins of *P. falciparum* blood stage Ags (rAgs) were used. (i) The 19-kDa C terminus of merozoite surface protein 1 ($MSP1_{19}$) was a fusion protein produced in baculovirus in *Sodoptera frugiperda*-infected insect cells; the sequence used corresponds to that of the E-KNG Palo Alto Uganda allele (28). The four allelic $MSP1_{19}$ variants (Q-KNG, E-KNG, Q-TSR, and E-TSR) were produced in the yeast *Saccharomyces cerevisiae* (27). (ii) Three allelic forms of MSP1 block 2, a polymorphic MSP1 domain located close to the N terminus, were cloned and expressed in *Escherichia coli* as glutathione transferase (GST) fusion proteins (26). The three variants were derived from the Palo Alto FUP, Wellcome, and Ghana RO33 strains and represent the K1, MAD20, and RO33 families, respectively (31). (iii) Two MSP2-derived rAgs, MSP2-2CD4 and MSP2-2CH4, representing the two major allelic families (3D7 and FC27, respectively), were expressed in *E. coli* as GST fusion proteins and consisted of the N- and C-terminal dimorphic sequences of MSP2 without the polymorphic repeat sequences. Neither the conserved sequences nor the polymorphic repeats were included in the proteins (15). (iv) R23 and PfEB200 were derived from red blood cell-associated Ags R23 (derived from gene R45) and PfEB200 (a subdomain of the giant protein Ag 332) described previously (3, 29). They were produced as proteins fused to GST in *E. coli*.

PBMC preparation and cell cultures. PBMCs were prepared as described previously, and T, B, and non-T, non-B cells were enumerated and characterized by means of flow cytometry, as described previously (21). The concentration of PBMCs was adjusted to 10^6 cells/ml in Iscoves' Dulbecco modified medium (Gibco BRL, Paisley, Scotland) supplemented exactly as described previously (21). The batch of fetal calf serum used (HyClone, Logan, Utah) was selected because it did not support spontaneous immunoglobulin production by B cells or promote differentiation of B cells (21). To test for viability and functionality of the cell populations, PBMCs were exposed to either anti-μ-chain Ab fragments (10 μg/ml; Immunotech, Marseille, France) or *Staphylococcus aureus* Cowan I strain-inactivated particles (1/25,000; Calbiochem, San Jose, Calif.) for 10 days in the presence of 50 IU of interleulin-2 (IL-2) (Sanofi, Labège, France) per ml (16). PBMCs were cultured with or without Ag in the presence or absence of cytokines (IL-2 [Sanofi]; IL-10 [Schering-Plough, Dardilly, France]; or IL-4, IL-1β, or IL-6 [PeproTech, London, United Kingdom]) at various concentrations or in the presence or absence of soluble recombinant trimeric CD40L (sCD40L, CD154) molecules (Immunex, Seattle, Wash.) or anti-CD40 monoclonal antibody (MAb) (clone 89; Schering-Plough). To test for spontaneous production of IgG in vitro, a cycloheximide control (100 μg/ml; Sigma, St. Louis, Mo.) was included for each individual cell culture.

Individual PBMC samples were cultured with various concentrations of crude parasite extract or rAgs. The optimal concentration for each stimulus was defined as the concentration giving rise to the maximum IgG concentration in in vitro cultures. The duration of PBMC cultures was also optimized on the basis of the maximum IgG production for a given type of stimulation.

ELISA detection of specific IgGs in individual plasma samples and in culture supernatants. Plasma samples from immune and nonimmune individuals were tested for Abs to crude *P. falciparum* Ag extract or for reactivity to individual Ags by ELISA as described previously. IgM and IgG subclasses were tested as described previously (2, 10, 33).

Culture supernatants were recovered and tested for polyclonal or Ag-specific IgGs by ELISA as described previously (17, 19, 39). Polyclonal peroxidase-conjugated goat anti-human IgGs were obtained from Cappel-Organon Technika, (Turnhout, Belgium) and used at the following dilutions: total IgG, 1:6,000; IgG1, 1:2,000; IgG2, 1:10,000; IgG3, 1:10,000; and IgG4, 1:30,000. Orthotoluidine-H_2O_2 (1:1, 100 μl; Sigma) was added, and each preparation was incubated for 20 min at room temperature; this was followed by addition of 100 μl of 0.1 N HCl to stop the reaction, and the absorbance was read at 450 nm. To minimize day-to-day, plate-to-plate, and/or isotype-to-isotype variation, data were expressed as the ratio of the optical density to the optical density of the appropriate control (GST for GST-fused proteins; insect cell culture medium or culture medium for the other proteins) (21). Optical density ratios of ≥1.5 (corresponding approximately to the mean ± 3 standard deviations) were considered positive (21, 39).

Statistical analysis. The frequency of responders was compared by using the Fisher exact test (χ^2). Data for different groups were compared by using the Mann-Whitney U test. Statview 5 software (SAS Institute, Cary, N.C.) was used.

RESULTS

PBMCs from the individuals from Dielmo and Ndiop, where *P. falciparum* is endemic, produced antimalarial Abs in vitro in response to stimulation with crude *P. falciparum* Ag. The ratio of T cells to B cells in PBMC preparations was approximately 7:1, with minimal interindividual variation. The number of non-T, non-B cells in the various PBMC preparations did not differ significantly between donors (data not shown). We tested for the presence of natural Ab bound to PBMCs (especially to monocytes) by preculturing the cells for 24 h. No *P. falciparum*-specific Ab was found in the culture supernatants regardless of the previous *P. falciparum* exposure of the donor, indicating that natural Abs either were not present or were degraded.

Each individual's PBMCs were then tested for viability and for the capacity to undergo polyclonal B-cell stimulation and differentiation and to produce immunoglobulin in vitro. To do this, PBMCs from individuals from areas where *P. falciparum* is endemic and controls were exposed to anti-μ-chain Ab fragments or *S. aureus* Cowan I strain for 10 days in the presence of IL-2, and the total IgG was measured in culture supernatants. PBMCs from the vast majority of donors (91.4%) proved to be capable of producing IgG (data not shown). This indicated that under the culture conditions used, the cells tested were viable and functional.

When cultivated in the presence of crude schizont Ag alone, the PMBCs from only 5 of 35 (14.3%) of the immune donors who were exposed to *P. falciparum* throughout their lives produced specific IgG (Table 1). Addition of IL-2 (50 IU/ml), IL-10 (100 IU/ml), IL-6 (50 to 200 IU/ml), or IL-1β (50 to 200 IU/ml) alone only minimally affected this proportion. In contrast, addition of IL-2 plus IL-10 resulted in a significant increase in the proportion of responders ($P < 0.001$). There was no additional increase when IL-6 or sCD40L was added together with IL-2 plus IL-10. Culture of *P. falciparum* crude

TABLE 1. Specific in vitro IgG responses for PBMCs from immune individuals in response to a crude *P. falciparum* schizont extract

Culture conditions[a]	No. (%) of responding individuals ($n = 35$)
Cytokines only	0
Ag only[b]	5 (14.3)[c]
Ag + IL-2	8 (22.9)
Ag + IL-10	10 (28.6)
Ag + IL-6	3 (8.6)
Ag + IL-1β	3 (8.6)
sCD40L only	0
Ag + sCD40L	3 (8.6)
Ag + IL-2 + IL-10	24 (68.6)[d]
Ag + IL-2 + IL-10 + IL-6	23 (65.7)[d]
Ag + IL-2 + IL-10 + sCD40L	23 (65.7)[d]
Ag + IL-4	21 (61.5)[e]
Ag + IL-4 + sCD40L	22 (64.7)[d,e]

[a] Optimal culture conditions were determined after various concentrations of cytokines were tested in preliminary assays.
[b] Crude schizont lysate of parasitized erythrocytes grown in culture.
[c] A responding individual was an individual for whom the ratio of the optical density of the culture to the optical density of the appropriate control was ≥1.5.
[d] Values are not statistically different.
[e] $n = 34$.

extract-stimulated PBMCs with exogenous IL-4 instead of IL-2 plus IL-10 also led to production of IgG in 61.5% of the individual cultures. Addition of cytokines at the onset of the cultures was required for any effect on IgG production to be seen (data not shown). None of the cytokines tested alone or in combination induced specific Ab production in vitro (Table 1). Neither addition of the sCD40L trimer (1 ng/ml) (Table 1) nor addition of the anti-CD40 MAb (1 μg/ml) (data not shown) significantly changed the proportion of Ab producers. Of note was the finding that neither sCD40L alone (Table 1) nor anti-CD40 MAb alone (data not shown) induced specific Ab production in vitro.

To investigate the influence of preexposure to *P. falciparum* on specific IgG in vitro production, we compared PBMCs from individuals recruited in different areas where malaria is endemic (Table 2). No IgG production was detected in PBMCs from European expatriates with no history of *P. falciparum* infection. Specific IgG production in vitro was rarely observed in individuals from the Dakar hypoendemic area. In contrast, large proportions of PBMCs from individuals exposed throughout their lives from Dielmo (a holoendemic setting) or from Ndiop (a mesoendemic village) produced *P. falciparum*-specific IgG in vitro. There was no significant difference between these two groups. Similar findings were observed with merozoite-enriched extracts (data not shown). Gender did not affect the responses (data not shown). Based on these observations, the villagers from the two settings were considered members of a single group in subsequent analyses.

In vitro IgG response to stimulation with *P. falciparum* rAgs. PBMCs were stimulated with rAgs derived from conserved ($MSP1_{19}$, R23, PfEB200) or polymorphic (MSP1 block 2-K1, -MAD20, and -RO33; MSP2-2CH4 and -2CD4) *P. falciparum* blood stages.

In vitro production of Ab to at least one rAg was observed in PBMCs from 68.8% of the Dielmo and Ndiop donors but not in PBMCs from the Dakar residents and the unexposed donors (data not shown). When PBMCs were stimulated with any rAg in the presence of IL-2 and IL-10, Ab responses were barely observed. However, costimulation with sCD40L in addition to IL-2 and IL-10 resulted in most cases in consistent Ab production.

No production of Ab to the conserved R23 and PfEB200 rAgs and to the polymorphic MSP2-2CH2 rAg was observed in the absence of CD40/CD40L signaling (Table 3). CD40/CD40L signaling did not significantly increase the percentage of responders to the polymorphic MSP1 block 2 Ags but strongly enhanced production of Ab to the conserved $MSP1_{19}$ rAg ($P < 0.002$). In these cultures, there was no difference in the production of specific IgG when the costimulus was sCD40L or anti-CD40 MAb (data not shown). In vitro Ab production was compared with the Ab present in each individual's plasma at the time of blood sampling for 15 Dielmo and Ndiop donors. The results are shown in Table 4. The concordance of in vitro production and in vivo production was highly Ag dependent, even for conserved Ags. PBMCs from 9 of the 11 individuals with plasma Ab to the $MSP1_{19}$ Ag produced Ab to $MSP1_{19}$ in vitro, but three of four plasma-negative individuals also produced $MSP1_{19}$-positive cultures. For R23, in vitro Ab production was observed for 7 of 11 plasma-positive individuals and for one of four plasma-negative donors. In contrast, cells from only 1 of 10 individuals with plasma Ab to PfEB200 produced anti-PfEB200 Abs in vitro. Importantly, the discordant scenarios observed with R23 and PfEB200 indicate that the GST carrier does not play a critical role in in vitro Ab production.

For polymorphic Ags, concordance between in vivo and in vitro responses was observed in approximately 50% of the

TABLE 2. Influence of preexposure to *P. falciparum* infection on the specific in vitro IgG responses to a crude *P. falciparum* extract

Culture conditions	No. (%) of responding individuals			
	Exposed individuals recruited in:			Nonimmune European expatriates ($n = 7$)
	Holoendemic area ($n = 19$)[b]	Mesoendemic area ($n = 16$)[b]	Hypoendemic area ($n = 15$)[b]	
Spontaneous	0	0	0	0
Ag only[a]	3 (15.8)[c]	2 (12.5)	0	0
Ag + IL-2 + IL-10	12 (63.2)[d]	11 (68.8)[d]	2 (13.3)	0

[a] Crude schizont lysate of parasitized erythrocytes.
[b] The holoendemic area was Dielmo village, the mesoendemic area was Ndiop village, and the hypoendemic area was Dakar.
[c] A responding individual was an individual for whom the ratio of the optical density of the culture to the optical density of the appropriate control was ≥1.5.
[d] Values are not statistically different.

TABLE 3. Influence of a CD40 stimulus on in vitro specific IgG production for a *P. falciparum* crude extract in the presence of IL-2 and IL-10

Ag used[a]	Protein concn (μg/ml)	No. (%) of responding individuals with the following costimuli/no. tested	
		None	sCD40L
None		0/35	0/35
$MSP1_{19}$[b]	0.01	2/20 (10)	12/20 (60)
R23	0.1	0/10	4/10 (40)
PfEB200	0.01	0/10	4/10 (40)
MSP2-2CH4	0.01	0/10	2/10 (20)
MSP1 block 2-RO33	0.1	3/15 (20)	4/15 (26.7)
MSP1 block 2-MAD20	0.1	4/15 (26.7)	4/15 (26.7)
MSP block 2-K1	0.1	4/15 (26.7)	10/15 (66.7)

[a] Cultures were grown in the presence of IL-2 and IL-10.
[b] Recombinant MSP2 variant. $MSP1_{19}$ was produced in baculovirus-infected insect cells.

cases. PBMCs from four of seven, three of six, three of four, and two of four plasma-positive subjects produced specific Abs to MSP2-derived Ags, MSP1 block 2-RO33, MSP1 block 2-K1, and MSP1 block 2-MAD20, respectively. Interestingly, apart from MSP1 block 2-K1, there was marginal in vitro production of a specific Ab in the absence of in vitro-detectable Ab, indicating again that the GST moiety played a limited role. The proportion of individuals with no detectable plasma Ab was greater than the proportion for the conserved Ag studied, which is not surprising as the Ags used here represent only a subset of the Ag specificities identified for these polymorphic loci (31). Hence, the Abs detected here, in culture supernatants or in plasma, probably represent a fraction of the possible repertoire of Abs to the MSP2 or MSP1 block 2 polymorphic domains.

Isotype distribution for Ag-specific IgG subclass production in vitro. The isotypes of the specific IgGs produced in vitro were assessed. In 18 of 23 (78%) of the cultures stimulated with a crude *P. falciparum* extract, anti-*P. falciparum* Abs of multiple subclasses were detected (Table 5). There were significantly more multiple than single subclass responses under these culture conditions ($P < 0.002$). The most prevalent subclasses of Abs to a crude *P. falciparum* extract produced were IgG1, IgG3, and, to a lesser extent, IgG2. Importantly, IL-6, sCD40L, or anti-CD40 MAb did not significantly affect the IgG subclass distribution (data not shown).

In contrast, single subclass responses were observed for the conserved R23 or PfEB200 rAgs and the variant MSP1 block 2-RO33, MSP2-2CH4, and MSP2-2CD4 rAgs (Table 5). Multiple subclass responses were observed in some cultures stimulated with $MSP1_{19}$ and with the MSP1 block 2-K1 and MSP1 block 2-MAD20 rAgs. In the cultures producing more than one subclass, no more than two subclasses were detected, and the responses were mostly a combination of IgG1 and IgG3. There was one culture in which production of IgG2 and IgG4 was detected. Similar results were obtained when Ag-exposed PBMCs were costimulated with IL-4 and sCD40L (data not shown). In contrast to crude *P. falciparum* extract stimulation, there were more single than multiple subclass responses to R23 ($P = 0.006$) or PfEB200 ($P = 0.002$) and MSP1 block 2-K1 ($P = 0.05$) rAgs. The similar trend observed for the other rAgs was not statistically significant.

Since the conformation of $MSP1_{19}$ has been shown to influence both T- and B-cell responses to this Ag (1, 9, 13, 20), we investigated whether the expression system used to produce $MSP1_{19}$ influenced IgG subclass production in vitro. PBMCs from seven donors were stimulated with baculovirus and yeast products expressing the same allele. For four donors, the IgG subclass of the Abs produced was the same for both forms of the Ag; however, PBMCs from one donor produced an IgG1 and IgG3 response to the baculovirus product but only an IgG3 response to the yeast protein, and PBMCs from another donor responded only to the baculovirus preparation (data not shown). Whether these findings were chance events or reflected the effects of subtle differences in conformation and/or posttranslational modification of the protein is unknown.

We next examined whether $MSP1_{19}$ variants induced similar or different profiles of Abs in vitro. Four variants (Q-KNG, E-KNG, Q-TSR, and E-TSR) of the yeast $MSP1_{19}$ Ag were tested. Three of four donors responded consistently to all the variants tested, with minimal differences in subclass distribution. One donor responded only to a single variant and produced IgG2 (data not shown). These data suggest that sequence variation has a limited effect on the frequency or subclass of the IgG response in vitro.

The concordance of the in vitro-produced specific IgG subclasses with the subclasses of circulating plasma Abs was excellent for $MSP1_{19}$, R23, and MSP2, intermediate for MSP1 block 2, and very low for PfEB200 (Table 5). For $MSP1_{19}$, six of seven IgG1 and three of four IgG3 responses were concordant, but two individuals made IgG2 in vitro, whereas no IgG2 was detected in plasma. For R23, three of four IgG1, three of four IgG2, and one of two IgG3 Ab responses were concordant. For MSP2, four of five IgG3 responses were concordant, but two cultures did not produce IgG1, while this subclass was detected in the corresponding plasma. Approximately 35% of the in vitro and in vivo responses were concordant for MSP1 block 2 regardless of the subclass except for IgG2, which could

TABLE 4. In vivo IgG Abs relative to in vitro IgG Abs to conserved and polymorphic *P. falciparum* Ags in donors immune to *P. falciparum*

Status of immune donors	No. of Ag-specific IgG responses in individual donor plasma and PBMC culture supernatants						
	$MSP1_{19}$	R23	PfEB200	MSP2-2CH4	MSP1 block 2-RO33	MSP1 block 2-K1	MSP1 block 2-MAD20
Plasma positive, culture positive[a]	9	7	1	4	4	3	2
Plasma positive, culture negative[a]	2	4	9	3	2	4	2
Plasma negative, culture negative	1	3	4	8	8	5	10
Plasma negative, culture positive	3	1	1	0	1	3	1

[a] Plasma positive irrespective of the isotype responses in plasma.

TABLE 5. IgG subclass distribution of Abs produced in vitro by PBMCs of immune individuals stimulated with various *P. falciparum* Ags

Prepn	No. positive/no. tested														
	Single isotype responses				Mixed isotype responses										
	IgG1	IgG2	IgG3	IgG4	IgG1, IgG2	IgG1, IgG3	IgG1, IgG4	IgG2, IgG3	IgG2, IgG4	IgG3, IgG4	IgG1, IgG2, IgG3	IgG1, IgG2, IgG4	IgG1, IgG3, IgG4	IgG2, IgG3, IgG4	IgG1, IgG2, IgG3, IgG4
Crude Ag extract[a]	3/35	0/35	2/35	0/35	1/35	5/35	0/35	0/35	0/35	0/35	7/35	1/35	0/35	0/35	4/35
rAgs															
$MSP1_{19}$[b]	3/15	3/15	0/15	0/15	0/15	3/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15
R23	3/15	2/15	1/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15
PfEB200	0/15	3/15	4/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15
MSP2-2CH4	0/15	0/15	4/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15
MSP1 block 2-RO33	2/15	1/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15
MSP1 block 2-K1	2/15	3/15	1/15	2/15	0/15	1/15	0/15	0/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15
MSP1 block 2-MAD20	1/15	1/15	1/15	0/15	0/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15

[a] Cultures were grown with IL-2 and IL-10 and in the presence (rAgs) or the absence (crude Ag extract) of sCD40L.
[b] The $MSP1_{19}$ used was the baculovirus-insect cell product.

be produced in some cultures but was rarely found in the corresponding plasma.

Delineation of factors affecting the production of defined IgG subclasses. To delineate the effect of the cytokine environment on the production of specific IgG subclasses, a series of PBMC cultures were grown in the presence of sCD40L and either IL-2 plus IL-10 or IL-4. PBMCs from 10 donors were stimulated with the conserved $MSP1_{19}$, R23, or PfEB200 rAg and with one variant Ag (MSP2-2CH4). There was no significant difference in the frequency of the IgG1 and IgG3 responses or in the frequency of the IgG4 responses, although the frequency of IgG2 production was reduced in the presence of IL-4 (Fig. 1). Thus, the profile of IgG subclasses produced after stimulation of PBMCs by individual *P. falciparum* rAgs is minimally affected by the exogenous cytokine environment, except perhaps for IgG2.

To investigate the effect of the Ag itself on IgG production in vitro, we examined parallel cultures from 15 donors with three rAgs (the conserved $MSP1_{19}$ and R23 Ags and MSP1 block 2-RO33). Specific Ab production was observed for 12 of the 15 donors. The results are shown in Table 6. The Ab production patterns varied from donor to donor. Some donors (e.g., donors 1, 2, 6, 8, 10, 12, and 13) responded to a single Ag, with variable subclass distribution (although one IgG subclass was still produced in each culture except for certain cultures with $MSP1_{19}$, in which IgG1 and IgG3 were produced). Five donors responded to two Ags. Two donors (donors 4 and 9) responded by producing the same subclass of IgG to different rAgs. Importantly, in three of five cases, the IgG subclass distribution was Ag specific, with different subclasses of IgG to different Ags produced by PBMCs from the same donor. For example, PBMCs from donor 14 produced IgG1 in response to $MSP1_{19}$ and IgG2 upon stimulation with R23. Thus, the subclass of Ab produced in response to an individual Ag varied from donor to donor, but, importantly, for each donor the subclass of Ab produced was Ag dependent.

DISCUSSION

The purpose of this study was to develop and evaluate the in vitro production of malaria-specific Abs in order to identify the critical signals involved in driving malaria-specific B cells to produce specific immunoglobulin subclasses. Production of anti-*P. falciparum* Ab in vitro required T-cell-derived signals, such as IL-2 plus IL-10 (or IL-4), in agreement with studies of other systems (17, 19, 20, 22). We have shown that B cells from donors exposed to malaria, but not B cells from donors who are not exposed to malaria, can be reactivated in vitro to secrete malaria-specific Abs and that for many individuals the pattern of in vitro Ab production mirrors the pattern of circulating Ab in the plasma. Taken together, these data suggest that the assay that we have developed detects predominantly memory B-cell responses.

Crude *P. falciparum* extracts induced production of multiple IgG subclasses, whereas single purified *P. falciparum* rAgs induced more restricted Ab profiles with, characteristically, one or at most two IgG subclasses present. For some rAgs, there was a strong tendency for cells from all donors to produce the same IgG subclass (e.g., IgG1 plus IgG3 for $MSP1_{19}$, IgG2 or IgG3 for PfEB200, and IgG3 for MSP2), while for other rAgs

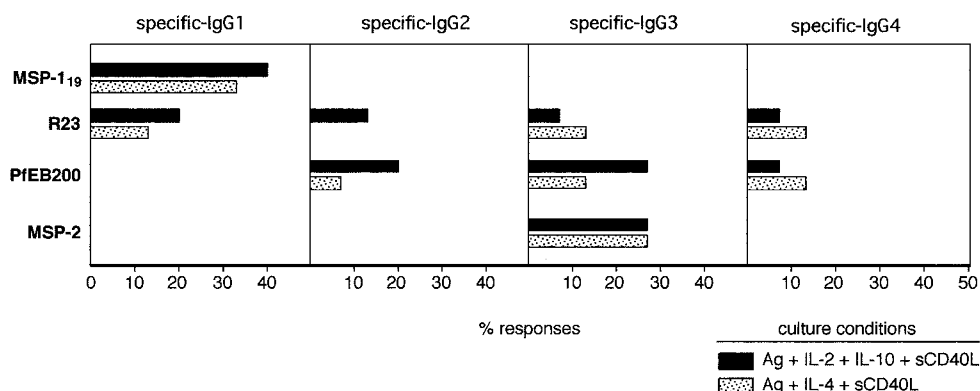

FIG. 1. In vitro production of IgG subclasses by PBMCs of immune individuals in response to various *P. falciparum* Ags. PBMCs (10^6 cells/ml) from 10 immune individuals were cultured for 12 days in the presence or absence of Ag at the optimal concentration and in the presence or absence of 50 IU of IL-2 per ml, 100 IU of IL-10 per ml, and 1 ng of sCD40L per ml (solid bars) or in the presence or absence of 100 IU of IL-4 per ml plus 1 ng of sCD40L per ml (stippled bars). Individual culture supernatants were tested for the presence of Ag-specific IgG1, IgG2, IgG3, and IgG4. The data are the percentages of the responses in individual cultures. Only optical density ratios greater than 1.5 were considered positive.

there was some heterogeneity between donors in the IgG subclasses produced in vitro. Importantly, cells from the same donor could be induced to produce Abs of different subclasses in response to different Ags. This indicates that the major factor determining the type of IgG produced seems to be the identity of the malaria Ag. The mixed-subclass response to parasite extract is thus most likely explained by the presence of many different proteins in the crude extract, each of which induces its own characteristic IgG response. The fact that the two ends of the MSP1 molecule (the N-terminal block 2 and the C-terminal $MSP1_{19}$) induced very different IgG responses in vitro indicates that there is domain-specific regulation of the response to this surface Ag. This is in line with recent studies of *Plasmodium chabaudi* (37).

Our results, however, do not give any indication of how the different Ags influence the IgG response. Neither the degree of conservation of the Ag nor the presence or absence of tandem repeats appears to play a critical role in determining the IgG subclass. We could find no consistent evidence that the cytokine milieu had an overriding effect on the IgG subclass, with the noticeable exception that IL-4 reduced the IgG2 responses. It thus seems unlikely that cytokines present during the restimulation of memory cells are able to alter preexisting switch patterns. Studies of other systems (16) suggest that the cytokine milieu may be crucial in determining switch patterns in naive B cells. It is possible that different malaria Ags induce different cytokine responses, which may influence class switching during the earlier stages of development of antimalarial immunity.

It is noteworthy that in vitro responses to R23, $MSP1_{19}$, and MSP2 rAgs paralleled plasma Ab profiles. In contrast, most plasma-positive donors failed to produce in vitro PfEB200-specific IgG, suggesting that an essential component is missing from the culture system and hence that regulation of the in vitro Ab response to this Ag may differ from regulation of the in vitro Ab responses to the other Ags studied.

For the three MSP1 block 2-derived Ags, the in vitro Ab profiles did not mirror the IgG3-skewed plasma profile (7, 26, 37). All four IgG subclasses were produced in vitro in response to these Ags. These three Ags are representatives of a very large population of polymorphic alleles to which donors may have been exposed. If the Ag used for restimulation differs in the precise T- or B-cell epitopes from the Ag that induced the observed plasma Ab response, it may be difficult for memory responses to be recalled in vitro, and the IgG produced in vitro may thus actually represent a primary in vitro response. The 10-fold-higher Ag concentration needed to promote in vitro IgG secretion compared to the concentrations of other recombinants may be indirect evidence of this in vitro priming. On the other hand, we have previously shown that the fine specificity of plasma Ab to MSP1 block 2 is stable over long periods of time despite reexposure to numerous variant MSP1 Ags (26). This suggests that there is an imprinted response that

TABLE 6. Ag-specific IgG subclasses produced in vitro by parallel cultures of PBMCs stimulated with distinct rAgs

Ag	IgG subclass(es)[c]											
	Donor 1	Donor 2	Donor 4	Donor 5	Donor 6	Donor 8	Donor 9	Donor 10	Donor 11	Donor 12	Donor 13	Donor 14
$MSP1_{19}$[a]	IgG2	IgG1, IgG3			IgG1	IgG1, IgG3	IgG1		IgG2		IgG1, IgG3	IgG1
R23[b]			IgG1	IgG2			IgG1	IgG3		IgG2		IgG2
MSP1 block 2-RO33[b]			IgG1	IgG4					IgG3			

[a] Produced in the baculovirus-insect cell system.
[b] *E. coli*-produced GST fusions.
[c] All cultures were grown with IL-2, IL-10, and sCD40L.

should effectively be recalled even when the recall Ag differs slightly from the inducing Ag.

In vitro IgG responses to MSP2 are entirely restricted to IgG3, which matches the skewness of naturally acquired plasma Abs to MSP2 (14, 41, 42). Interestingly, immunization of mice with recombinant MSP2 also induces a similarly skewed IgG response (IgG2b, the murine analog of human IgG3), and there is a complete lack of murine IgG1 (the major subclass that is typically produced in response to protein Ags in the mouse) (E. M. Riley, unpublished data). It is worth noting that the two allelic variants of MSP2 induced similar responses, suggesting that epitopes within the conserved domain of the molecule may drive the IgG3 switch. MSP2 is thus a useful model to study class switching to IgG3 in individuals exposed to malaria.

The in vitro Ab responses to $MSP1_{19}$ reflected the characteristic subclasses observed in vivo, namely, IgG1 alone or combined IgG1 and IgG3 responses (33, 38). This type of response was observed regardless of the expression system or the allelic variants used. We have reported previously that $MSP1_{19}$-stimulated memory-type B cells secrete IL-10 (17, 18). Whether induction of IgG1 and/or IgG1 plus IgG3 is associated with this IL-10 production is an open question. Elucidation of the factors contributing to this production of IgG1 and/or IgG1 plus IgG3 is important, as these subclasses have been shown to play a critical role in the Ab-based protection against parasite blood stages (5, 6, 23).

In summary, the work reported here delineated a specific set of conditions favoring in vitro production of Ab against several *P. falciparum* vaccine candidates by PBMCs from donors frequently exposed to *P. falciparum* infections. Data presented here indicate that intrinsic properties of the protein Ag itself play a major role in determining the subclass of the Ab response, which has important implications for rational design of vaccine delivery. Additional work is needed to identify the culture conditions allowing Ab production for nonimmune subjects, the target group for vaccination, in whom de novo class switching is likely to be achieved.

ACKNOWLEDGMENTS

We are indebted to physicians, nurses, health care officers, scientists, and technicians of the Dielmo and Ndiop projects, who provided clinical, parasitological, and entomological data used to carry out this study, and especially to C. Rogier (Institut Pasteur de Dakar) and J.-F. Trape, D. Fontenille, and L. Lochouarn (IRD, Dakar, Senegal). We are grateful to G. Michel and A. Samb (Hôpital Principal de Dakar) for providing blood samples from the Senegalese Army Blood Bank; to F. Brière (Schering-Plough) for providing IL-10 and MAb 89 to human CD40; to Elaine Thomas (Immunex, Seattle, Wash.) for providing sCD40L molecules; and to Sanofi for the kind gift of IL-2. We are indebted to S. Franks (University of Edinburgh) for cloning and expressing the MSP2 rAgs and to I. Holm and M. Guillotte (Institut Pasteur, Paris, France) for preparation of other rAgs. The technical expertise of M. B. Diouf in preparing the crude parasite Ag used in this study is also acknowledged.

This work was supported by grants from the Institut Pasteur and from the Ministère Français de la Coopération et du Développement, Paris, France. R23, PfEB200, and baculovirus $MSP\text{-}1_{19}$ were produced with support from the Institut Pasteur and CNRS (URA 1960); MSP-2 antigens were produced with support from the Wellcome Trust. G. M. Engler was a summer student sponsored by a Lucy E. Moten fellowship, Howard University, Washington, D.C.

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Editor: W. A. Petri, Jr.